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Review: The future of plant pathogen diagnostics in a nursery production system

Alka Rani[a], Nerida Donovan[b], Nitin Mantri[a,*]

[a] School of Science, The Pangenomics Group, RMIT University, Melbourne, VIC, 3083, Australia
[b] Elizabeth Macarthur Agricultural Institute, NSW Department of Primary Industries, Menangle, NSW, 2568, Australia

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ABSTRACT

Plant diseases have a global economic impact through the loss of productivity and trade restrictions. Production of disease free plants in nurseries is crucial for plant survival and productivity in the field environment. Accurate diagnosis of plant pathogens helps to identify appropriate management practices to reduce production losses. Current diagnostic methods for plant pathogens include evaluation of disease symptoms, identification of culturable organisms or direct analysis of infected tissue by serological and molecular methods. Laboratory methods can be laborious, expensive and require specific technical expertise. There is a strong demand for the development of rapid, specific, sensitive and cost-effective tests that can be used at the point-of-care in nurseries. This review summarises disease diagnostic methods that have been successfully applied in other fields, and have the potential to transform production in the nursery industry. Emerging technologies include isothermal amplification, nanomaterial-based detection, biosensors, robotics, lab-on-chip, and paper-based analytical devices.

1. Introduction

Nurseries are used to propagate plants of fruit, vegetable, forestry, and ornamental species. They serve as the backbone for many agricultural and horticultural production systems and are therefore of immense economic importance. In 2015–2016, 19,000 people were employed in Australian nursery production systems and 1.6 billion nursery plants were sold across Australia generating $2.29 billion AUD in revenue which has increased to $2.45 billion AUD in 2017–2018 (Nursery papers, 2018; Australian Horticulture I, 2019). In the US, nursery market revenue has increased by 4.3% in the last five years with annual growth of businesses and employees of 1.1% and 1.8%, respectively. The total revenue from nursery sales increased from $41 billion in 2006 to $48 billion in 2018 (Nursery and Garden Stores-US Market Research Report, 2018).

Global trade of nursery stock, even where strict quarantine practices are followed, creates a risk of trading diseases at the same time. During the 2017/18 year, Australian imports of nursery stock were valued at $37.7 million, with exports valued at $5.6 million (Australian Horticulture I, 2019). Nurseries are higly vulnerable to plant disease with the potential to cause significant economic impact within and beyond the nursery production system, particularly if the diseased stock is not identified prior to leaving the nursery. The import of infected plant material, including live plant stocks, into nurseries is thought to be responsible for approximately 69% of pathogen outbreaks worldwide (Liebhold et al., 2012). Several reports describe the spread of plant diseases from greenhouses to agricultural, horticultural and forestry land (Liebhold et al., 2012; Stewart-Wade, 2011; Parke et al., 2014). Consequential losses can be significant, as reported in the US state of Georgia where the loss due to plant diseases in 2015 was approximately $800 million, rising from $647.2 million in 2006 (Martinez-Espinoza, 2013). Examples of pathogen outbreaks resulting from the movement of nursery plants are provided in Table 1.

Nurseries differ from agricultural monocultures in that there are often many different types of plants co-located within a small area (Bagshaw Rogerset al., 2008) which can increase the number of potential pathogens in the system and make disease detection and management challenging. Pathogen infection may induce symptoms in plants or may be asymptomatic (latent) making it difficult to identify affected plants without formal diagnostic testing. Early detection of pathogens in a nursery can help prevent the spread of disease beyond the nursery environment. This requires rapid, specific, sensitive, and cost-effective diagnostic techniques that can be routinely used to check for notorious pathogens before the plants are sold or transported for large scale production (Bagshaw Rogerset al., 2008).

Conventional methods for detecting nursery diseases involve assessing the symptoms and host range, and identification of isolated organisms by microscopy or biochemical tests. These tests may be

* Corresponding author.
E-mail address: nitin.mantri@rmit.edu.au (N. Mantri).

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Table 1
Example of the common plant pathogens reported to emanate from the movement of nursery plants.

Pathogen	Type of pathogen	Specific Host	Reference
Phytophthora lateralis	Oomycete	ornamental and Port-Orford-cedars	Robin et al. (2011)
Citrus tristeza virus	Virus	Citrus scion and rootstocks	(Moreno et al., 2008; Bar-Joseph et al., 1989)
Cronartium ribicola	Fungus	white pine, currant, gooseberry	Jennifer and Parke (2012)
Phytophthora parsiana	Oomycete	fig, almond, pistachio	(Parke et al., 2014; Fichtner et al., 2016)
Cryphonectria parasitica	Fungus	chestnut and forestry species	Melissa Petruzzello and Pallardy (2018)
Phytophthora ramorum	Oomycete	Many plant forest and ornamental species including oak	Goss et al. (2011)
'*Candidatus* Liberibacter asiaticus' *(CLas)*	Bacteria (gram negative)	Citrus and other Rutaceous species	Neupane et al. (2016)
Ralstonia solanacearum Race 3 biovar 2	Bacteria (gram negative)	potato, tomato, eggplant, geranium, pepper, and many non-solanaceous plants	Champoiseau et al. (2009)
Rose rosette virus	Virus (single-stranded negative RNA)	rose	Olson et al. (2015)
Xanthomonas albilineans	Bacteria (gram negative)	sugarcane	Lin et al. (2018)
Macrophomina phaseolina	Fungus	More than 500 species including cabbage, chickpea, peanut, cauliflower, soybean, potato, sweet potato, sesame, wheat, and corn	Fuhlbohm et al. (2012)

reliable, but they are sophisticated to perform and require trained professionals. Additionally, they may exhibit low sensitivity, be time-consuming (Keremane et al., 2015; Coy et al., 2014; Morgan et al., 2012) and cannot be used for unculturable organisms. Serological methods, such as ELISA (enzyme-linked immunosorbent assay), based on the detection of a protein molecule using polyclonal or monoclonal antibodies, are broadly applied in plant nursery diagnostics. ELISA based methods include direct tissue blot immunoassay (DTBIA), double antibody sandwich (DAS) ELISA and tissue-print (TP) ELISA (Vidal et al., 2012; Ding et al., 2016). The dipstick ELISA test has been widely adopted in commercial nurseries due to the ease of use in the nursery at the point-of-care (POC) (Cambra et al., 2000). These methods have been found to be suitable for on-site, specific detection of many plant pathogens, but they may be less sensitive and have the potential for cross-reaction with non-target pathogens, particularly in multiplexed reactions (Djelouah et al., 2014).

A number of molecular approaches, based on the polymerase chain reaction (PCR) have been developed; the variants of PCR involve reverse transcription PCR (RT-PCR), real-time PCR (qPCR), immunocapture PCR (IC-PCR), and nested PCR. All have shown improved sensitivity and specificity over ELISA approaches to detect bacteria, viruses, and fungi (Coy et al., 2014; Lin et al., 2010, 2018; Yakabe et al., 2009; Capote et al., 2009; Sano et al., 1992). Despite the benefits, PCR based approaches also require sophisticated lab equipment and skilled operators. Moreover, the technology may have issues with specificity and be prone to false positive results by detecting even a small amount of contamination in the reaction mixture (Osterbauer and Trippe, 2005; Yamane et al., 2008). Table 2 compares techniques currently available for plant nursery diagnostics.

New high throughput diagnostic techniques are available and are being applied in other fields, but are yet to be adopted for nursery disease diagnostics. Fig. 1 provides a schematic overview of current and emerging techniques that are applicable for detection of nursery pathogens. A number of a recent reviews outline advanced diagnostic techniques for field pathogens, foodborne pathogens or clinical pathogens (Baron, 2011; Khater et al., 2017; Priyanka et al., 2016) but no current review focuses on the detection of nursery pathogens. This review describes advanced diagnostic techniques which may be used to improve on-site detection of plant pathogens in nurseries, minimising the impact of plant disease within and beyond the nursery environment. This review will assist both researchers and the nursery industry to innovate in the development of rapid, low-cost POC detection techniques with increased sensitivity and specificity.

2. Emerging high throughput diagnostic techniques

The new high throughput diagnostic techniques can be lab-based or point-of-care based (Fig. 2A). Table 3 compares emerging diagnostic techniques

2.1. Lab-based methods

2.1.1. Droplet Digital PCR

Droplet Digital PCR (ddPCR), developed in 2011 to increase the efficiency of conventional PCR (Hindson et al., 2011), is based on water-oil emulsion droplet technology where a sample is fractionated into 20,000 droplets, and PCR amplification of the template molecules occur in each individual droplet. ddPCR is precise, sensitive and robust, producing simple readouts such as 'YES' or 'NO'. It is an absolute quantification system which eliminates the need for standard curves and normalization. This high throughput system has the ability to perform approximately 2 million PCR reactions in a single 96 well PCR plate and read 32 plates per hour. The technology can also be multiplexed to analyze up to 10 multiple targets in a single run using multiple fluorescent labeled probes (Hindson et al., 2011). Interference due to the presence of inhibitors and contaminants is reduced, even in sub-optimal samples. Additionally, ddPCR has been found to be useful where targets are present at low levels or are unevenly distributed in infected plants (Selvaraj et al., 2018; Dreo et al., 2014).

However, in ddPCR, there is the risk of false positives where unequal distribution of genome in droplets affects the sensitivity of the test and the presence of one target in multiple droplets leads to an overestimation of pathogen levels. Dreo et al. (2014) proposed enzymatic digestion of DNA to improve target separation in droplets for accurate quantification. Adding bacterial culture into the reaction instead of extracting DNA also proved successful for detecting the target pathogen with fewer background signals (Dreo et al., 2014).

ddPCR has been reported to detect as low as 1 copy of a pathogen in 5 ml of lake water, 1 copy of peripheral blood mononuclear cell (PBMC) in 10,000 human cells and 2.2 copies of the plant pathogen '*Candidatus* Liberibacter asiaticus' (CLas) in a 20 μL reaction (Selvaraj et al., 2018). Until now, ddPCR has only been developed for a few nursery pathogens such as Grapevine red blotchy–associated virus, crown gall of grapevines caused by *Agrobacterium vitis* (Voegel and Nelson, 2018), Palm-infecting Phytoplasmas, *Ralstonia solanacearum,* and *Erwinia amylovora* (Dreo et al., 2014).

The method requires an expensive thermocycler and reagents which limits its use for on-site pathogen detection (Hindson et al., 2011; Dreo et al., 2014). Despite the drawbacks, ddPCR is widely used for analyses

Table 2
Comparison of conventional plant pathogen diagnostic methods.

Techniques	Tested Pathogen	Cost per reaction	Limit of detection	Sampling type	Instrument required	Time of Assay	Cross-reactivity	POC possibilities	Output type	References
Culturing	Bacteria, fungi	Variable, depends on media type	1–10^3 CFU/ml	infected plant material, sap or extract	Incubator, LAF desirable	Days to weeks	Moderate	No	qualitative	(Champoiseau et al., 2009; McClure and Brown, 2008; Pringsh, 1990)
DTBIA	Virus, bacteria, fungi	Variable, depend on Ab type	Not known	plant tissue typically stems, petioles, peduncles or ovaries	Shaker desirable	4 h	Moderate	Moderate	Qualitative	Djelouah et al. (2014)
ELISA	Bacteria, virus, viroids	Variable, depending on Ab type	1–10 ng/ml	Plant extract	Plate reader	2–3 h	Moderate	Yes	Semiquantitative	(Cambra et al., 2000; Maliogka et al., 2015)
Convention al PCR	Bacteria, Virus, viroids, Fungi	$6	10 pg/µl	Purified nucleic acid	Thermocycl er, GE equipment	1.5–2 h	Strong	No	Semiquantitative, qualitative	Goss et al. (2011)
RT-PCR	Virus, viroids	$9	8.64 × $l0^{-4}$ ng/nL	Purified nucleic acid	Thermal cycler, GE equipment	1.5–2 h	Strong	No	Semiquantitative, qualitative	(Vidal et al., 2012; Yamane et al., 2008)
Immunocap ture PCR	Bacteria, virus, fungi, viroids	$8	10 fg/µL	Plant extract	Thermal cycler,	3–4 h	Very low	No	Qualitative, semiquantitative	Sano et al. (1992)
Nested PCR	Virus, bacteria, fungi	$7	0.1 pg/µL	Purified nucleic acid	Thermal cycler, GE equipment	3–4 h	Very low		Semiquantitative, qualitative	Coy et al. (2014)
Real - time PCR	Bacteria, virus, fungi, viroids	$7	1 fg/HL	Purified nucleic acid	Real-time thermal cycler	2 h	Low	No	Relative- quantitative, qualitative	(Li et al., 2009; Tooley et al., 2006)

LAF- laminar air flow, **GE**-gel electrophoresis.

Table 3
Comparison of emerging diagnostic techniques.

Techniques	Tested pathogen	Cost per reaction	Limit of detection	Sample type	Instrument required	Time of Assay	Cross-reactivity	POC possibilities	Output type	References
LAMP	Bacteria, fungi, virus, viroids	$3	0.5 ag	Purified nucleic acid	Water bath, agarose GE	60–80 min	Low	Moderate	Semiquantitative	(Keremane et al., 2015; Notomi et al., 2000)
Droplet digital PCR	Bacteria, fungi, virus	$5	200 fg	Purified nucleic acid	Droplet generator, thermal cycler, droplet reader, centrifuge	3–4 h	Low	No	Absolute quantitation, qualitative	Selvaraj et al. (2018)
RPA	Virus, Bacteria, Fungi, viroids	$6	Ifg	Purified nucleic acid	GE equipmen t	20–30 min	Very low	Yes	Qualitative, semiquantitative	(Babu et al., 2017; Ghosh et al., 2018)
SERS	Bacteria, virus, fungi, viroids	$7-8	1.9 fg	Purified nucleic acid	SERS spectroph otometer or microsco pe	1–2 h	Very low	Yes	Qualitative, semiquantitative	Lau et al. (2016)

GE-gel electrophoresis.

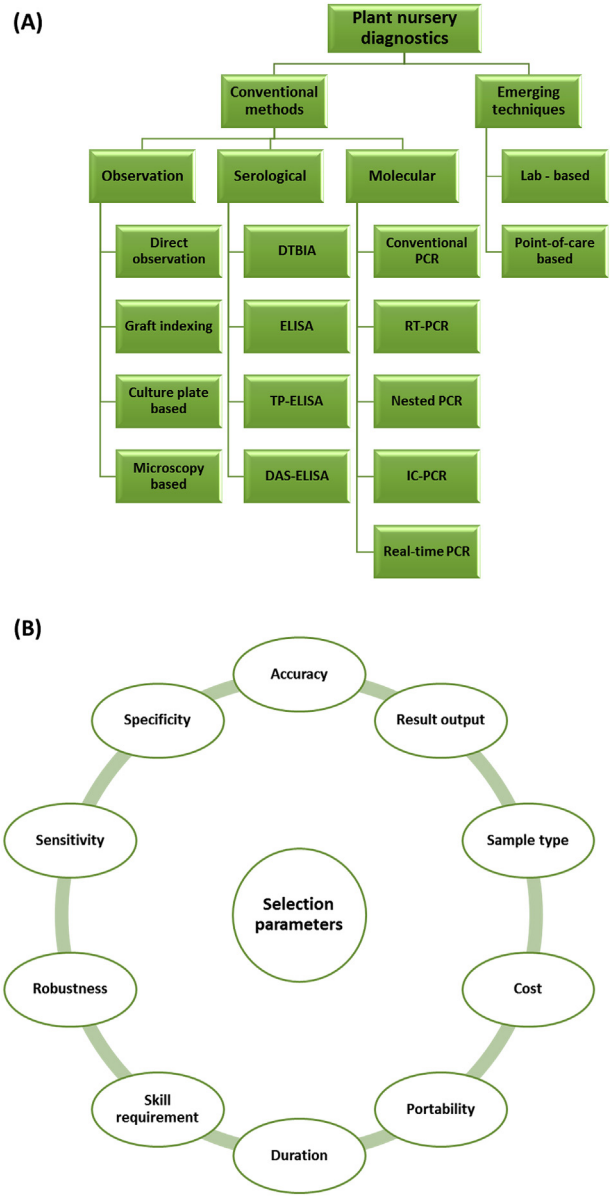

Fig. 1. Schematic illustration of (A) current and prospective detection methods for the plant nursery diagnostics and (B) their parameters of selection.

of microbiomes, copy number variation (CNV), gene expression, environmental monitoring, food testing, clustered regularly interspaced short palindromic repeats (CRISPER) workflow, and pathogen detection (Selvaraj et al., 2018; Hindson et al., 2011). Due to its broad applicability and sensitivity, ddPCR is well suited to laboratory-based detection of nursery plant pathogens.

2.1.2. Surface-enhanced Raman spectroscopy

Surface-enhanced Raman spectroscopy (SERS) is an intrinsic signal amplification technique that is based on a non-catalytic physical phenomenon i.e. localized surface plasmon resonance (LSPR) of a nanostructured metal surface. The interaction between the electromagnetic radiation and a metallic surface excites the LSP and in turn generates an amplified electric field (EF) around a nanosized rough surface. This results in resonance in the visible and near-infrared regions of the electromagnetic spectrum. The Raman peaks are usually 10–100 times narrower than any fluorescent peaks. Different types of Raman active reporter molecules (RRM) produce peaks at different spectrums with little overlap making SERS suitable for multiplexed detection with high sensitivity. When RRM is co-immobilized on a nanoparticle then it is called an extrinsic Raman label (ERL) (Granger et al., 2016).

SERS has been widely applied using immunoprobes and molecular probes for pathogen monitoring in humans, plants, animals, food, water, and the environment due to its rapidity, sensitivity, and robustness for detection of biomarkers (Granger et al., 2016). Clinical pathogens, such as *Escherichia coli, Enterococcus lactis, Morganella morganii,* and *Lactobacillus casei* have been successfully detected using a label-free SERS method within 5 min (Dina et al., 2017). In addition, a nano-dielectrophoretic (DEP) enrichment strategy has been integrated with SERS reporters to build a microfluidics, self-referencing diagnostic platform with the ability to detect 10 CFU/mL of mixed strains of *E. coli* with more than 95% accuracy (Wang et al., 2017).

Detection of the plant pathogen *Phytophthora ramorum* using label-free SERS was first reported in 2015 after amplifying DNA from infected *Rhododendron* leaves. Silver NPs were synthesized onto a glass substrate along with a biotin-labeled DNA probe and horseradish peroxidase (HRP) conjugated with streptavidin which produced a coloured readout after capturing the target molecule (Yüksel et al., 2015). SERS also proved to be a suitable method for the multiplex detection of agriculture-related pathogens such as *Botrytis cinerea, Pseudomonas syringae,* and *Fusarium oxysporum*. The assay was able to detect as low as 1 copy of pathogenic DNA (Lau et al., 2016). This sensitivity indicates the strong potential of SERS for lab-based nursery diagnostics. Recently, companies like Thermofisher Scientific have developed hand-held Raman SERS instruments that are routinely used for forensic inspection (Doty and Lednev, 2018) and environmental regulators. Although these handheld devices are less powerful than a large lab based SERS machine, they can still be helpful for detection of plant pathogens in the field. There have been reports of using these handheld Raman SERS instruments for detection of human, animal and plant pathogens (Egging et al., 2018) with the lowest LOD being 10 ng/mL (Owens et al., 2018) with 100% accuracy (Sanchez et al., 2019; Farber and Kurouski, 2018).

2.2. Point-of-care (POC) based methods

POC approaches can be performed by non-experts for on-site detection of pathogens without the need for sophisticated lab equipment. The sale of such devices reached an estimated $US 23.71 billion in 2017 which is predicted to increase to $US 38.13 billion by 2022 (https://www.marketsandmarkets.com/PressReleases/point-of-care-diagnostic.asp). POC devices have advantages for use in nursery diagnostics including portability, sensitivity, specificity, robustness, ease of handling and high throughput ability.

2.2.1. Isothermal amplification methods

Isothermal amplification was a breakthrough in the field of molecular diagnostics, eliminating the need for a thermocycler to amplify nucleic acid. Many isothermal techniques have been applied to plant diagnostics such as loop-mediated amplification (LAMP), rolling circle amplification (RCA), nucleic acid sequence based amplification (NASBA), helicase-dependent amplification (HDA), strand displacement amplification (SDA), recombinase polymerase amplification (RPA), Single Primer Isothermal Amplification (SPIA), isothermal multiple displacement amplification (IMDA), multiple displacement amplification, and nicking and extension amplification reaction (NEAR) (Table 4). Most of these are chiefly combined with microfluidics, microarray, slip chip and spinning disc to enhance the applicability and efficiency of assays in various fields (Dimov et al., 2008; Shen et al., 2011). In plant diagnostics, LAMP and RPA have prominently been used due to their broad range of application and rapidity and, therefore these have the most potential for plant nursery diagnostics (Zhao et al., 2015).

2.2.1.1. Loop-mediated amplification (LAMP) method. LAMP is a target

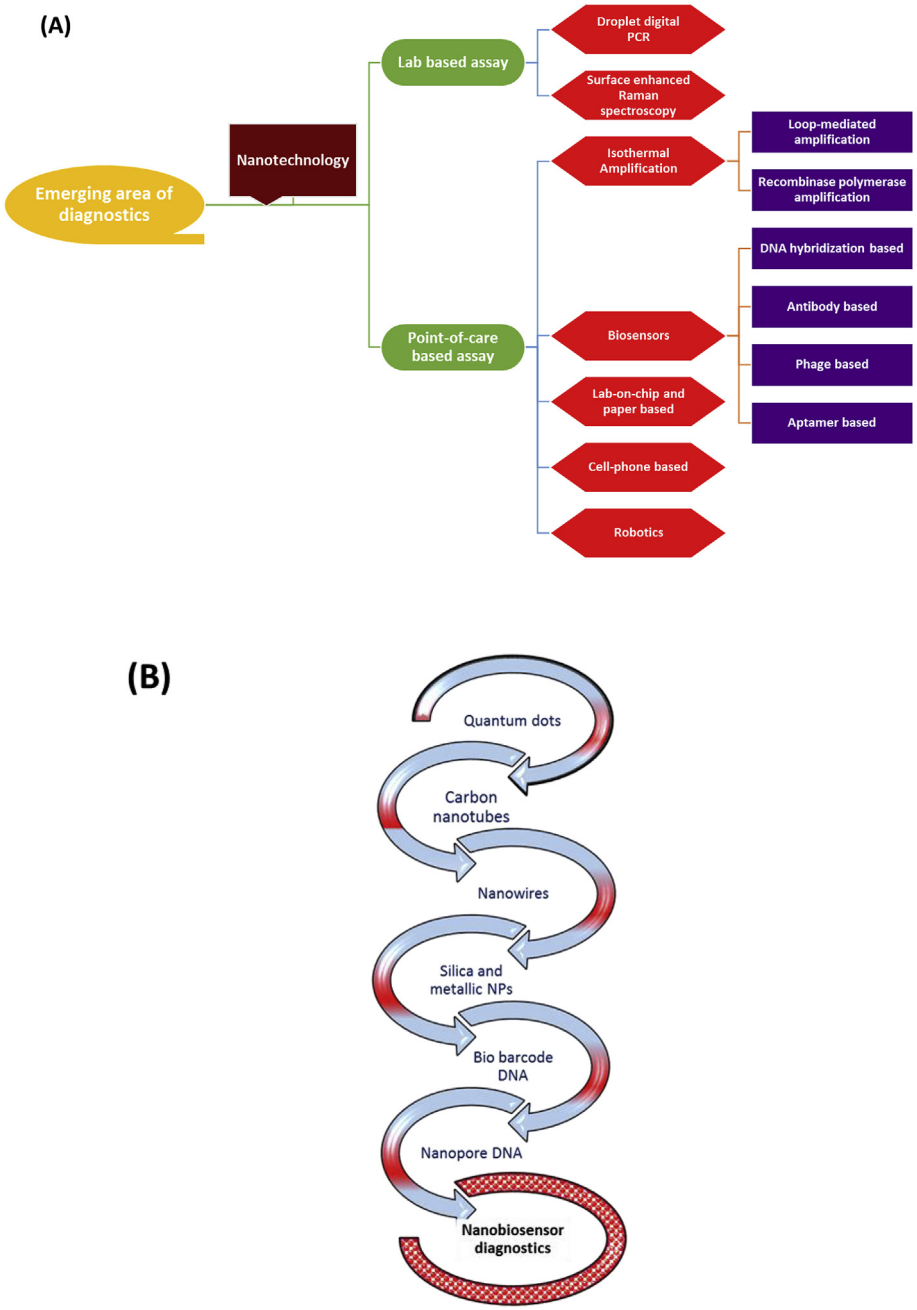

Fig. 2. Overview of the listed new emerging techniques (A) and list of nanotechnology-based material used for biosensing (B).

amplification method which uses six primers highly specific to eight different target sites in the target sequence (Notomi et al., 2000). LAMP is suitable for DNA genomes and RNA genomes with the addition of RTase enzyme (RT-LAMP). The modification of LAMP using a fluorescent probe makes it a real-time isothermal amplification method which delivers quick and simple absolute quantification of pathogenic DNA (Tomita et al., 2008; Zhang et al., 2013).

However, LAMP has some limitations such as the need for a heat block or hot water bath to maintain a temperature of 65 °C, and design of the 6 primer pairs. There is also a high risk of primer dimer formation (Fukuta et al., 2003). The potential benefits lending this technology to nursery diagnostics are ease of use, high specificity, and robustness,

Table 4
Comparison of different isothermal techniques.

Technique	RPA	LAMP	RCA	NASBA	HDA	SDA	NEAR	MDA	SPIA
Time	20–40 min	50–60 min	90 min	90–120 min	90 min	90–120 min	20–30 min	18–24 h	3–4 h
Enzymes involved	3	1	1	3	2	2	2	2	3
Temperature (°C)	37–42	65	37	41	37	37	55	30	45–50
Primers	2	8	1	2	2	4	2	2	1
Type of template	DNA, RNA	DNA, RNA	Circular DNA, RNA	RNA	DNA, RNA	DNA, RNA	DNA, RNA	DNA, RNA	DNA, RNA

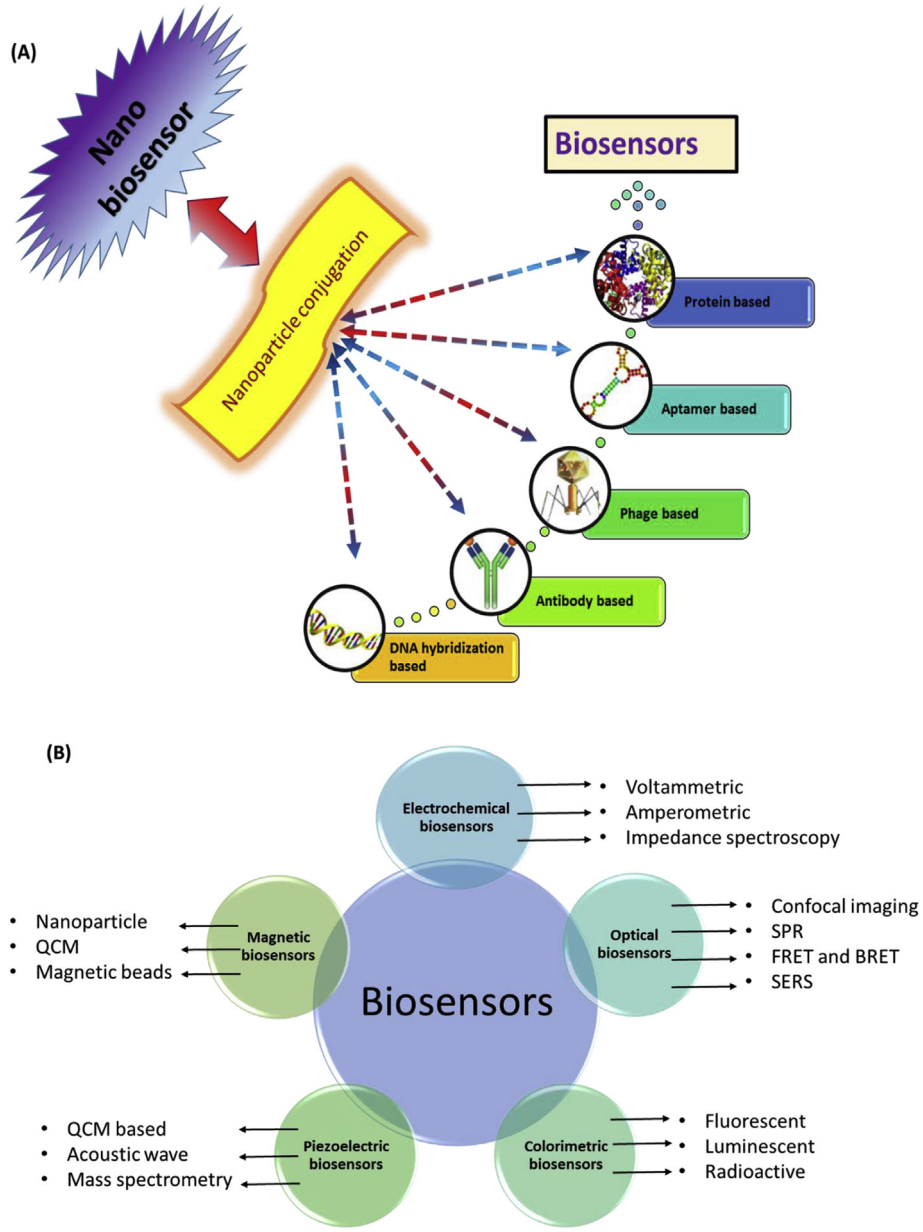

Fig. 3. Types of biosensors based on the type of recognition moiety of target pathogen (A) and based on the type of transducer used for detection of bio molecule (B).

even in the presence of contaminants and inhibitors. LAMP has been reported to be 100 times more sensitive and simple than qPCR and can amplify 10^9 copies of DNA in less than 1 h (Notomi et al., 2000; Wu et al., 2016). LAMP has gained significance in the last few years for plant nursery diagnostics, with reported sensitivity up to autograms or 5–6 copies of target DNA (Wu et al., 2016) and has been used for detection of '*CLas*', *E. amylovora and P. ramorum* (Keremane et al., 2015). The LAMP assay has also been combined with a lateral flow device for the detection of *Xylella fastidiosa* in olive plants. Detection kits are commercially available but a lack of information on robustness, sensitivity, and specificity of these costly kits limits their use for routine plant pathogen diagnostics (Djelouah et al., 2014).

2.2.1.2. Recombinase polymerase amplification (RPA). RPA is the most recent isothermal technique and has the advantage of amplification at ambient room temperature, although maximum output occurs at 37–42 °C. RPA is performed by three enzymes, recombinase, single-stranded DNA binding protein (SSB) and strand displacing polymerase and uses primers of 32–36 nucleotides in length. In the presence of an ATP molecule, a recombinase enzyme scans the double-stranded target sequence to bind primers on cognate sites and open the double helical structure which is stabilized by the SSB protein. ATP hydrolysis facilitates the disassembly of recombinase and then a strand displacing polymerase adds the complementary nucleotides into the primer sequence to form a new strand of DNA (Piepenburg et al., 2006; Daher et al., 2015).

Although loopholes of RPA arise with DNA extraction and primer design, the effectiveness of RPA is strongly affected by the length of the primers. Daher et al. reported reduced efficiency of RPA with a mismatch sequence at the 3' end primer (Daher et al., 2015). According to another report, a mismatch of 12% of bases reduced DNA heteroduplex formation by 50% and the reaction stopped when the percentage of mismatched bases increased to 15% (Patil et al., 2011). To reduce the complication associated with the DNA extraction, Babu et al. (2017) and Qian et al. (2018) attempted RPA with crude plant extract, but outputs were inconsistent.

Despite these shortcomings, RPA has gained interest in diagnostics due to its rapidity, sensitivity, selectivity and low cost. RPA has the

capability to detect fewer copies of DNA in 30 min and multiple targets in one reaction (Babu et al., 2017). RPA can be performed as reverse transcription RPA (RT-RPA) and as a lateral flow RPA (LF-RPA) assay. The technology has been used for on-site detection of plant pathogens such as *P. syringae,* Cucumber mosaic virus (CMV), *Agrobacterium* spp., Plum pox virus (PPV) and *CLas* (Ghosh et al., 2018; Wee et al., 2015; He et al., 2014).

The RPA assay is also suitable for paper and plastic based LF devices, nanotechnology-based devices, microfluidics-based devices, and biosensors (Rohrman and Richards-Kortum, 2012; Sun et al., 2016). An RPA based electrochemical biosensor proved 10,000 times more sensitive than conventional PCR to detect *P. syringae* in asymptomatic infected plant tissue when integrated with gold nanoparticles (Lau et al., 2017). Another variant is digital RPA, which is carried out on a slip chip and was developed for methicillin-resistant *Staphylococcus aureus* (MRSA) detection. Digital RPA gives output as presence or absence (as 0 and 1) by detecting a change in fluorescence intensity if the target is present. One advantage is that 1,000 reactions can be performed simultaneously (Shen et al., 2011). RPA can also be conjugated with surface-enhanced Raman spectroscopy (SERS) to enhance the signal quality; this combination has been reported to detect as low as 1 copy of a plant pathogen such as *B. cinerea, P. syringae and F. oxysporum* (Lau et al., 2016). Development of RPA assays for plant pathogens to be used at the POC in nurseries has great potential, particularly for early detection of exotic pathogens during an incursion.

2.2.2. *Biosensing approaches*

In the field of diagnostics, biosensing approaches are replacing traditional detection methods due to their small size of unit and portability. Targets can be a whole antigen, released toxin, mycotoxin or nucleic acid. The biosensors are classified according to their receptors' moiety (Fig. 3 A) or the type of transducer used for signal generation (Fig. 3 B) (Singh et al., 2013; Bahadir and Sezginturk, 2016; Fang and Ramasamy, 2015). Electronic signals are generated after processing the biological sample, transduction of signals and amplification of signals (Fig. 4).

2.2.2.1. DNA hybridization based biosensors. DNA based biosensors work on the principle of complementary sequence hybridization between the immobilized probe and the target DNA sequence. The probe is an artificially synthesized complementary DNA sequence designed to capture the target sequence. RNA targets can also be detected using reverse transcriptase (RTase) enzyme which converts the RNA into cDNA prior to hybridization. Subsequently, transducers convert the sensed DNA target into the signals which are further increased by an amplifier (Fig. 5). Signal generation can occur by electrochemical, optical, magnetoelectric, piezoelectric and colorimetric reactions, depending on the type of transducer used. Labels can be redox, fluorescent, enzyme, chemiluminescent or nanoparticle. Nucleic acid-based biosensors are very stable in a liquid state for rapid, sensitive and cost-effective identification of a pathogen (Zhai et al., 1997).

DNA based electrochemical biosensors relying on a redox reaction, transfer electrons between electrodes after hybridizing the target to an immobilized capture probe, whereas electrochemical label-free biosensors depend on the change in impedance, current or conductivity (Liu et al., 2012; Liu et al., 2017; Batchelor-McAuley et al., 2009). An electrochemical voltammetric biosensor has been developed to diagnose sugarcane white leaf disease (caused by a phytoplasma) using a carbon glass electrode and methylene blue as an indicator (Wongkaew and Poosittisak, 2014).

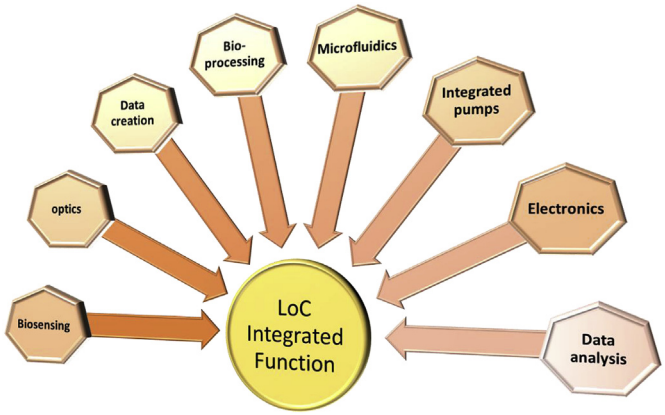

Fig. 5. Integrated functions of a lab-on-chip device.

Over the past decade, NPs have been used to tag amplified targets for easy visualization of the product. Chua et al. (2011) developed a glass fibre-based DNA biosensor for the detection of *Vibrio cholerae*, a foodborne clinical pathogen, using gold NPs attached to the detection reagent. They successfully detected 5 ng of pathogen DNA visually, which eliminated the need for agarose gels and carcinogenic DNA dyes like ethidium bromide (Wongkaew and Poosittisak, 2014; Chua et al., 2011). Another RT-electrochemical genosensor was developed to detect 12.8 pg/mL of PPV in greenhouse plants (Malecka et al., 2014). Lau et al. proved DNA biosensors were 10,000 times more sensitive than conventional PCR; being able to detect 13 pg of *P. syringae* in asymptomatic plant tissue. RPA was used to amplify the target DNA and colloidal gold nanoparticles were attached to the detection probes for signal enhancement (Lau et al., 2017).

However, these biosensors have pitfalls. The requirement for pure nucleic acid and its amplification can be challenging given nucleic acid extracts are prone to contamination and degradation. There is also the risk of false positive or negative results and the inability to differentiate between live and dead pathogen or toxins. Additionally, piezoelectric and enzymatic DNA based biosensors are extremely sensitive and are readily affected by a change in the pH of the surroundings (Batchelor-McAuley et al., 2009). However, the coupling of biosensors with a DNA extraction dipstick (Zou et al., 2017), NPs and an isothermal amplification technique has prospects for effective on-site detection of nursery plant pathogens.

2.2.2.2. Antibody-based biosensors. These biosensors are based on the principle of ELISA for signal production after capturing of target antigen (Ag) by immobilized antibodies (Abs) on a solid surface made up of glass, plastic or a paper. The Ag-Ab complex can be separated by an immuno-magnetic separator (Cho and Irudayaraj, 2013). The signal generation depends on various types of potent transducers used such as optical, electrochemical, piezoelectric and surface plasma resonance (SPR). The detection can be label based or label-free (Pilolli et al., 2013), however, this technique is expensive, and the synthesis of antibodies can take a long time. The Ag-Ab reaction is readily influenced by changes in the physiological or biochemical conditions. There is also the possibility of cross-reactivity and false positive

Fig. 4. General steps conducted by biosensors to produce readable outputs.

outputs. Immunosensors possess a low shelf life creating the need to store them at a low temperature and detection is only qualitative, not quantitative (Yi-Xian et al., 2012; Lau and Botella, 2017).

Despite the limitations, these biosensors are broadly applied in many diagnostic fields as they do not require the extraction of the purified analyte. Graphene oxide (GO) (acting as a label) based immunobiosensors have been developed for the detection of rotavirus based on the phenomenon of fluorescence resonance energy transfer (FRET). When the Ab-DNA-AuNP complex binds to the target Ag that was linked to the GO arrays, photoluminescence quenching occurred between GO and AuNPs resulting in a reduction of fluorescence, indicating the presence of the pathogen (Jung et al., 2010; Liu et al., 2011).

The immuno-AuNP based biosensor, integrated with sample concentration and immuno-magnetic separation was developed to detect *E. coli* and *Salmonella typhimurium* within 2 h with the LOD 3 CFU/mL and 15 CFU/mL respectively (Cho and Irudayaraj, 2013). Another study demonstrated increased sensitivity of an immunosensor with nanofluidic concentration using a bead-based assay to detect the complex pathogen. Their study proved that a pre-concentration step can enhance the sensitivity 500 times (Wang and Han, 2008).

For plant pathogen diagnostics, an electrochemical impedance spectroscopic (EIS) immunobiosensor has been developed for CTV detection, based on the measurement of pulse voltammetric signals on the gold electrode (Haji-Hashemi et al., 2017). Electrochemical immunobiosensor incorporated with metallic NPs and horseradish peroxidase (enzyme as a label) have been developed where an enzyme performs a catalysis reaction followed by an amperometric or voltammetric redox reaction. This was shown to be 20–50 fold more sensitive than conventional ELISA to detect plant pathogens *Pantoea stewartii* (7.8×10^3 CFU/mL) (Zhao et al., 2014), and *Citrus tristeza virus* (CTV) (Shojaei et al., 2016). Ab-based biosensors, due to their enhanced efficiency and sensitivity, have prospects for use in nursery plant diagnostics.

2.2.2.3. Phage-based biosensors. Bacteriophages are DNA or RNA viral genomes which are encapsulated by a protein coat. They infect and multiply in the bacterial cells. Previously, phage were used to treat bacterial plant infections; however due to their binding affinity to bacterial cells, proteins or carbohydrates, phage have been used to diagnose infection. The interaction between phage and the targeted bacterial component brings about a change in the reaction which is converted into measurable signals by the transducer. The phage-based biosensors are very sensitive, specific, cost-effective, rapid, and robust and they exhibit a long shelf life due to their stability at a high temperature. These biosensors are capable of enumerating viable pathogens (Yue et al., 2017).

Drawbacks include the significant sample preparation required and their limited applicability for the detection of fungal and unculturable bacterial pathogens. Additionally, it has been reported that a lack of knowledge of phage-based biosensors exists (Singh et al., 2013). However, phage-based biosensors have been developed for the detection of foodborne pathogens. Yue et al., 2017 (Yue et al., 2017) synthesized a label-free electro-chemiluminescent (ECL) phage-based biosensor to detect 56 CFU/mL of *P. aeruginosa* in less than 30 min. They used PaP1 phage extracted from hospital sewage. The surface of a glass carbon electrode was fabricated through the deposition of phage-conjugated carboxyl graphene to capture *P. aeruginosa*. The ECL signals were decreased by an increase in the concentration of *P. aeruginosa* due to the formation of a non-conductive complex which blocks the interfacial electron transfer, therefore stopping the diffusion of the ECL active molecule (Yue et al., 2017).

For plant pathogen detection, phage-based magnetoelastic (ME) biosensors have been developed for the detection of *S. typhimurium* on the surface of tomato and spinach leaves. The limit of detection showed by this sensor was 1.94 CFU/leaf (Park et al., 2013a, 2013b). Despite the limited studies in this area for detection of plant pathogens, these phage-based biosensors have proven to be more sensitive and stable than real-time PCR (Park et al., 2013a), suggesting their potential use and further exploration for nursery plant diagnostics.

2.2.2.4. Aptamer-based biosensors. Aptamers, the polymers of single-stranded nucleic acids or amino acids of length 30–32 bp, exhibit high affinity to the target molecule which can be a whole cell or a small component of the cell such as a toxin, protein or an enzyme. The aptamer is selected and enriched by many iterative rounds of systematic evolution of ligands by exponential enrichment (SELEX) from an aptamer combinatorial library (containing approximately 10^{15} different sequences). An aptamer-based approach has significant benefits over the use of antibodies because of their low cost and reduced synthesis time (2 days) compared to Ab synthesis (3–4 months). Additionally, aptamers are also highly stable, flexible and have versatile binding capacities (Seok Kim et al., 2016).

Aptamer-based biosensors (aptasensors) involve the immobilization of a selected aptamer on a solid surface to capture the target molecule, which converts the resultant signal into a readout by the transducer. Aptasensors are compatible with various detection systems such as electrochemical, colorimetric, chemiluminescence, fluorometric, surface plasma resonance (SPR), magnetic and optical. Use of various nanomaterials (Fig. 2 B) in aptasensors has been proposed for increased sensitivity and specificity (Khedri et al., 2018). However, limitations exist such as the inefficiency to target small molecules, and the inability to find small differences between the large molecules and their limited applicability towards a diverse range of pathogens (Yi-Xian et al., 2012). Additionally, the traditional SELEX process is time-consuming, expensive, labour-intensive and inefficient. However, the new high throughput microfluidics-based automatic SELEX and graphene oxide-based screening approaches overcome the bottlenecks of traditional SELEX (Lin et al., 2014).

Zhang et al. reported an aptasensor with SPR and QCM detection systems which was highly sensitive; able to detect 0.1 aM of human thrombin when combined with NPs to enhance signal generation. They integrated their aptasensor with isothermal amplification (Zhang et al., 2014). Another apta nanobiosensor has been developed with the integration of graphene oxide (GO) on a microfluidics biochip to detect the clinical pathogens *Lactobacillus acidophilus*, *Staphylococcus aureus,* and *Salmonella enterica*. The detection was performed in 10 min and detected 11 CFU/mL (Zuo et al., 2013). Fang et al. developed a DNA extraction free lateral flow aptasensor to detect 10 colonies of *Salmonella enteritidis* at the POC using strand displacement isothermal amplification (Fang et al., 2014). Aptamer-based biosensing have been successfully applied to a wide range of clinical and foodborne pathogens: for example Influenza A and B viruses, human immunodeficiency virus −1 (HIV -1), Hepatitis C virus, *E. coli* strains DH 5 α, *Mycobacterium tuberculosis, Bacillus anthracis*, *Streptococcus pygenes,* and *Bacillus thuringiensis* (Yi-Xian et al., 2012).

β-conglutin, which is an allergen produced from the plant, *Lupinus albus* was detected using an aptasensor in 20 min with a LOD 8 fM. The study was conducted using duo aptamers, Fe_3O_4 @Au core-shell nanoparticles, AuNP, horseradish peroxidase (HRP), 3, 3′, 5, 5′-tetramethylbenzidine (TMB) and external magnetic separation. The basic principle involved was the capillary action of analytes toward the fabricated reagents and probes. When the analytes bound to the AuNP (conjugated with secondary aptamer) and were captured by the primary aptamer to form an aptamer-antigen-aptamer complex, a reaction occurs between HRP and TMB to produce a blue colour on the test line, whereas free AuNP produces a red colour on the control line (Wu et al., 2018).

In agriculture, these biosensors have not been widely used for plant pathogen detection. Balogh et al., 2010 developed the first aptamer against a plant viral coat protein; their targets were two strains (MT32 and PSA-H) of apple stem pitting virus (ASPV). The assay, double

oligonucleotide sandwich –enzyme-linked oligonucleotide assay (DOSELONA) was further developed for exploitation of the aptamer reaction through western blots and SPR (Balogh et al., 2010). The versatile nature of aptasensors and their portability lends them to use in plant pathogen diagnostics in nurseries. Potentially, a portable aptasensor integrated with nanoparticles may be a good option for disease detection in nurseries prior to any plant symptom expression (Seok Kim et al., 2016).

2.2.3. Lab-on-chip devices and paper-based devices

Lab-on-chip (LOC) is a miniaturized microfluidic device (mm to cm^2). It integrates multiple laboratory functions onto a single chip, including biochemical operations, chemical synthesis, DNA sequencing and pathogen detection (Fig. 5). The chip can be composed of glass, silicon, thermoplastic or polymer (poly-dimethylsiloxane or poly-methylmethacrylate) and requires complex fabrication. Advances of micro-electro-mechanical systems (microfluidics) and micro total analysis systems facilitate the integration of microelectronic and micromechanical structures into a single system. The different modules of a LOC device are the processor (for sampling), transducer (produces a measurable signal), amplifier (for signal amplification) and electronic software (for data analysis to reproduce readable outcomes) (Luka et al., 2015).

These devices use less sample volume (less than pL) and reagents to perform rapid microbial detection. The integration of NPs in a LOC device has proved more sensitive than ELISA assays with the ability to detect 0.1 pg/μL and 10^3 CFU/mL of *Phytophthora infestans* and *X. fastidiosa,* respectively (Chiriacò et al., 2018; Zhan et al., 2018). LOC devices also face challenges due to the requirement for new stable polymers and label-free assays which limits their use for on-site pathogen detection (Julich et al., 2011).

However, the development of microfluidics paper-based analytical devices (μPADs) with 2D & 3D potential have overcome the bottlenecks associated with conventional LOC devices by using a permeable porous cellulose paper (pore size 1–10 μm) which also reduces the cost. These devices carry tremendous ability to handle a large number of fluids. The μPADs work on the principle of capillary action or lateral flow. These are portable, disposable, simple, cheap and easy to handle. The main body of these devices is designed with four parts; the sample pad (to load the sample) composed of cellulose, a conjugate pad (for the binding of sample and label), an indicator pad (for the test and control line) and an absorption pad (for absorption of leftover fluids). Immobilization of various molecules become feasible due to the presence of many hydroxyl (-OH) and carboxylic (-COOH) groups on the cellulose (Mahato et al., 2017).

The first paper-based dipstick assay (immunochromatographic strip) was developed in 1950 to detect glucose in urine. Initially, μPADs only produced colorimetric readouts (qualitative) but later, with advances in wireless connectivity and hardware-software capabilities (Novarum, iBG star, iPhone, Android app), quantitation of the target can be performed using a cell phone (CP) based detection system or a small portable scanner (Syedmoradi et al., 2017). Paper-based immunostrips are commercially available to detect *Phytophthora* spp. and *R. solanacearum* for in-field use (Champoiseau et al., 2009). They have some limitations such as non-specific adsorption, light scattering, non-uniform wicking, and variable sensitivity. However, further advancement in μPADs can potentially lead to the development of a 'POCKET SIZED' diagnostic platform for detection of pathogens in nurseries (Mahato et al., 2017; Martinez, 2011).

2.2.4. Cell phone based (CP) based devices

CP based devices represent integrated systems of modern communication and sensor technologies to capture images (using an inbuilt camera), store the data and compare with the stored memory for images of other diseased plants. The data generated by these devices is easily transmissible from one place to another. CP-based detection platforms have gained importance for clinical use to monitor a range of parameters such as blood sugar, blood pressure, heart rate, sleep duration, body weight, and physical activity. A few applications and datasets have also been developed for plants, enabling pathogen detection based on color intensity, texture, spot count, and edge contour of the leaves. These observations feed to transformation circuits (RGB channels, gradient magnitude, gradient directions, intensities, local binary pattern of intensities and magnitudes) which compare the captured images with a stored dataset by constructing a statistical model (Neumann et al., 2016).

The plant disease version 2.3 app, developed by Nikos Petrellis attained a 90% success rate in recognition of disease in grapes (Petrellis, 2017). Mohanty et al., 2016 developed a Plantvillage dataset containing 54,306 images of healthy and infected plants covering 26 disorders in 14 different crops. This model has reportedly achieved 99.35% accuracy in finding infected plants (Mohanty et al., 2016). Plantix is the Google app established by a German-based AgTech startup that has more than 50,000 pictures of plants and it is able to detect more than 60 plant diseases (http://www.fao.org/e-agriculture/news/plantix-app-detect-and-cure-your-plant-diseases). Development of CP based devices has also been attempted for detection of assorted pathogens such as *P. syringae, Cercospora beticola, Uromyces betae, Puccinia triticina, Blumeria graminis* and *Septoria tritici* in wheat and sugarcane (Neumann et al., 2016). These methods require further development and optimization before their adoption in nursery diagnostics to replace molecular and serological techniques, especially in terms of specificity. However, these approaches are suitable for high throughput detection with the ability to categorise a large number of healthy and unhealthy traits in plants, suggesting a future role in plant nursery diagnostics (Vashist et al., 2015).

2.2.5. Robotic devices

Robotics represents a new approach in the field of plant disease detection. An autonomously guided vehicle can be used as a robotic diagnostic system in a controlled manner. The locomotory system is built to carry a navigator, sensor and other required tools to move the robot in designated rows in the field for automatic sampling and disease biosensing. Such systems have been developed through the synergetic combination of mechanical, electronic and electrical systems. Disease detection by robotics can either be direct sensing of plant disease with an electronic nose system or an indirect comparison of images of plants captured by an inbuilt camera. In direct sensing, an electronic nose determines the change in volatile organic compounds due to biotic or abiotic stress. In the indirect method, captured images are automatically digitally compared with stored memory to categorise the disease based on observed characteristics such as their colour, texture, morphology, and structure (Dudareva et al., 2006; Holopainen and Gershenzon, 2010).

The robotics developed by Srivastava et al., in 2010, named 'Robo Kisan', has proven satisfactory for the detection of downy mildew disease of grapes in the fields of India (Srivastava and Sharma, 2010). Similarly, image processing-derived automatic robotic detection systems have also been proposed for the detection of pathogens in pomegranate, citrus, and apple. Integrated systems using robotics are also being trialed where automatic remedial spraying is triggered upon detection of a pathogen (Kadam and Shukla, 2014; Sadistap et al., 2013; Patil and Kumar, 2011). Robotic systems alone, or in combination with bionanosensors, show great promise for development as automatic high throughput diagnostic systems at POC in plant nurseries (Awate et al., 2015).

3. Summary and conclusions

In the last few decades, increased inter- and intra-continental trade of nursery plants has escalated the spread of nursery-borne plant pathogens. Therefore, pathogen detection in nursery plants is critical prior

to export into agricultural and forestry production systems, commercial and home garden landscaping and the natural environment. Early detection in nurseries helps with disease management, reduces disease spread and increases the chance of successful eradication when new pathogens are introduced into a country or region. Conventional techniques for confirming the identity of plant pathogens in symptomatic nursery plants include plate culturing, biological indexing, ELISA and PCR. These assays have proven sensitive, specific and reliable to some extent, but have significant shortcomings such as the requirement for sophisticated equipment and skilled operators which precludes most conventional assays from POC use. Though portable dipstick ELISA assay has been developed for in-field detection of plant pathogens, there are issues with sensitivity and specificity (i.e. only genus specific) (Cheng et al., 2010).

Therefore, the development of sensitive and specific advanced diagnostic techniques will enable early detection (even in asymptomatic plants) and reduce the impact of plant pathogens in nursery production systems. In this article, new high throughput lab-based and point-of-care techniques and devices, were reviewed for their prospective use in nurseries. Lab -based techniques such as ddPCR and SERS are expensive, non-portable and require trained operators. However, they are specific and sensitive which allows for efficient use by biosecurity officers or industry resgulators to stop transport of infected material, preventing pathogen spread or entry into a new region. Multiplexing of assays to detect several pathogens simultaneously is also a major advantage of emerging lab-based methods. In comparison, point-of-care techniques such as isothermal amplification, biosensors, nanobiosensors, cell phone (CP) based devices, paper-based devices (PBD), lab-on-chip (LOC), and robotics are easy to use by a non-skilled person, require less handling and provide simple readouts. Ongoing research has improved their LOD and specificity, however, low sensitivity of some POC methods is still a bottleneck to identifying low levels of a pathogen in asymptomatic nursery plants. Importantly, POC methods are not as high throughput compared with emerging lab-based methods. However this limitation can be overcome by further improvements to the technology and integration of new technologies. For example Robotic nose or Robotic imaging using “drones” can be used to survey hundreds of nursery plants, with potential to modify the technology to spray the required pesticide upon detection of a pathogen in the nursery.

CRediT authorship contribution statement

Alka Rani: Writing - original draft. **Nerida Donovan:** Conceptualization, Writing - review & editing. **Nitin Mantri:** Conceptualization, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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